SNI

Standar Nasional Indonesia

SNI 3747:2009

Cocoa powder

ICS 67.140.30

National Standardization Agency of Indonesia

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BSN
Gd. Manggala Wanabakti
Blok IV, Lt. 3,4,7,10.
Telp. +6221-5747043
Fax. +6221-5747045
Email: dokinfo@bsn.go.id
www.bsn.go.id

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Foreword

Indonesian National Standard (SNI) *Cocoa powder* is a revision of SNI 01-3747-1995, *Cocoa powder.* The purpose of the formulation of this standard is:

- Protecting the health of consumers;
- Ensure fair and responsible trade of food products;
- Diversification of products / product development;
- Support the development of cocoa industry;

This standard is formulated by considering the following:

1. RI Law No. 7 Tahun 1996 on Food
2. RI Law No. 8 Tahun 1999 on Consumer Protection.
3. Government Regulation No. 69 / 1999 on Food Labels and Advertising
4. Decision Directorate General of Drug and Food No. 03725/B/SK/VII/89 on Maximum Metal Contamination Limits in Food and Beverages or its revision.
5. Decision Directorate General of Drug and Food No.03726/B/SK/VII/89 on Maximum Microbial Contamination Limits in Food and Beverages or its revision.

This standard was formulated by Technical Committee 67 - 04 Food and Beverages. This standard has been discussed in technical meetings and agreed on in a consensus meeting dated January 22, 2007 in Jakarta. The meeting was attended by representatives from consumers, manufacturers, testing agencies, Science and Technology Institution and other related agencies.

This standard has been through the process of polling from June 24, 2009 to of August 22, 2009 with RASNI as result.

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Cocoa powder

1 Scope

This standard specifies terms and definitions, quality requirements, sampling and test methods of cocoa powder.

2 Normative references

SNI 19-0428-1998, Petunjuk pengambilan contoh padatan (S*ampling instructions of solids).*

3 Terms and definitions

3.1

cocoa powder

product obtained from cocoa cake transformed into powder

3.2

cocoa press cake

product obtained from fat reduced cocoa powder with or without alkaline treatment

3.3

cocoa powder

product in the form of paste obtained from cocoa nib (cocoa pieces) through the milling without fat removal

3.4

cocoa nib

cocoa beans without shell

3.5

cocoa

seed of the cacao tree *(Theobroma cacao L.)* which has been cleaned and dried

3.6

alkalify

the process of adding a suitable alkaline material to cocoa beans or cocoa nibs or cacao press cake aimed to regulate the acidity in order to achieve the desired level

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4 Quality requirements

Quality requirements of cocoa powder is specified in Table 1 below.

Table 1 - Quality requirements of cocoa powder

Test parameters	Units	Quality requirements
Condition:		
a. Odor	-	typical cocoa, free from foreign smell
b. Taste	-	typical cocoa, free from foreign smell
c. Color	-	brown or other color due to alkalifying
Fineness (pass 200 mesh sieve) (w / w)	%	min. 99.5
Shell calculated from the alkaline free nib (w / w)	%	max. 1,75
Water content (w / w)	%	max. 5,0
Fat content (w / w)	%	min. 10,0
Metal contamination:		
a. Lead (Pb)	mg / kg	max. 2,0
b. Cadmium (Cd)	mg / kg	max. 1,0
c. Tin (Sn)	mg / kg	max. 40
Arsenic (As) contamination	mg / kg	max. 1,0
Microbial contamination:		
a. Total Plate Count	colonies / g	max. 5×10^3
b. Coli form bacteria	APM / g	< 3
c. Escherichia coli	per g	negative
d. Salmonella	per 25 g	negative
d. Mould	colonies / g	max. 50
e. Yeast	colonies / g	max. 50

5 Sampling methods

Sampling methods shall be in accordance with SNI 19-0428-1998.

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6 Test methods

Test method of cocoa powder is as follows:

a) Test sample preparation in accordance with Appendix A.1
b) Test of conditions in accordance with Appendix A.2
c) Fat content test in accordance with Appendix A.3
d) Water content test in accordance with Appendix A.4
e) Fineness test in accordance with Appendix A.5
f) Shell test in accordance with Appendix A.6
g) Test methods of metal contamination accordance with Appendix A.7
 - Lead (Pb) and cadmium (Cd) contamination test in accordance with Annex A.7.1
 - Tin (Sn) contamination test in accordance with Annex A.7.2
h) Arsenic contamination test in accordance with Appendix A.8
i) Microbial contamination test in accordance with Appendix A.9
 - Total plate count in accordance with Annex A.9.1
 - Coli form bacterial and E. coli test in accordance with Annex A.9.2
 - Salmonella test in accordance with Annex A.9.3
 - Mould / Yeast test in accordance with Appendix A.9.4

7 Acceptance requirements

Products are considered to have passed the test if they comply to all quality requirements according to Article 3.

8 Hygiene

Hygienic production of products, include its preparation and handling shall comply with the applicable provisions of the Guidelines for good manufacturing of processed food.

9 Packaging

Product shall be packed in a sealed container, not affected by or affecting its content, safe for storage and transportation.

10 Marking

Marking shall comply with the applicable regulations on food labels and advertising.

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Appendix A
(normative)
Test methods of cocoa powder

A .1 Preparation of chemical test samples

A .1.1 Determination

a) Mix the sample until evenly distributed, then make a square shaped sample of it and divide diagonally into four parts;
b) Take two parts opposite each other, mix until homogenuous, then repeat as above until a sufficient quantity to be analyzed is obtained;
c) Place the sample in a bottle and close the lid tightly.

A .2 Condition

Condition test in done by organoleptic testing of smell, taste, and color.

A .2.1 Odor

A .2.1.1 Principle

Conducted an analysis of the organoleptic test samples by using the nose for smelling

A .2.1.2 Determination

a) take 30 ml of the test sample that has been melted in a glass beaker;
b) smell test sample to find out its smell;
c) the work should be done by at least 3 trained panelists or more.

A .2.2 Flavor

A .2.2.1 Principle

Conducted an analysis of the organoleptic test samples by using the tongue to sense the taste.

A .2.2.2 Determination

a) take 50 ml of the test sample that has been melted in a glass beaker;
b) taste test sample to find out its taste;
c) the work should be done by at least 3 panelists or 1 trained panelist.

A .2.3 Color

A .2.3.1 Principle

Conducted an analysis of the organoleptic test samples by using the naked eye.

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A .2.3.2 Determination

a) take 50 ml of the test sample that has been melted in a glass beaker, pour into a cler white flask;
b) observe the test sample color;
c) the work should be done by at least 3 panelists or 1 trained panelist.

A.3 Fat content

A .3.1 Principle

Extract free oil from the sample of cocoa mass using non-polar organic solvents previously subject to hydrolysis.

A .3.2 Apparatus

a) analytical balance the a precision of 0.0001 g;
b) 250 ml capacity flat bottom boiling flask;
c) electric oven;
d) electric bath;
e) complete Soxhlet extraction equipment;
f) lead extraction or cartridge filter paper size 33 mm x 80 mm;
g) 300 ml - 500 ml glass cup;
h) watch glass;
i) fat-free filter paper;
j) dessicator.

A .3.3 Reagents

a) 8 M hydrochloric acid (HCl);
b) petroleum ether, boiling point 30 ° C - 60 ° C;
c) 0.1 N $AgNO_3$ silver solution

A .3.4 Determination

A .3.4.1 Hydrolysis

a) weigh up to 4 g 5 g of sample precisely into the glass cup;
b) slowly add 45 ml of boiled distilled water while stirring until homogeneous;
c) add 55 ml of 8M HCl (2 parts HCl plus 1 part water) and some boiling stones;
d) close the glass beaker with a watch glass and boil gently for 15 minutes;
e) wash with 100 ml of distilled water and pour the rinse water into the beaker;
f) filter the sediment using fat free filter paper;
g) rinse the glass beaker 3 times with distilled water, rinse until free from chlorine that can be determined by adding 1 drop up to 3 drops of the $AgNO_3$ ito the filtrate, if no white precipitate (AgCl) is formed it is free of chlorine;
h) replace the filter paper and contents into the lead extracitor or fat-free filter paper cartridge and dry for 6 hours to 18 hours at a temperature of 100 ° C to 101 ° C.

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A.3.4.2 Fat Extraction

a) dry boiling flask containing a few boiling stones for 1 (one) hour at a temperature of 100 ° C. Cool and weigh , connect to *Soxhlet* extraction equipment

b) then place the lead extractor or cartridge filter paper into the Soxhlet, and pour petroleum ether solvent until less than 2 / 3 capacity of the boiling flask. Then the extract for 4 hours and evaporate the solvent with an evaporator or it can be removed by heating the flask above a water bath.

c) Then dry boil flask and fat in the oven at a temperature of 100 ° C for 1.5 hours up to 2 hours, cool in a dessicator and weigh;

d) repeat drying until the difference in fat weight made in succession is less than 0.05%.

A .3.5 Calculation

Fat percentage expressed in weight percentage and is calculated in dry weight using the following formula:

$$\text{Fat (\%)} = \frac{(m_1 - m_2)}{m_0} \times 100\%$$

where:

m_1 is the weight of flask + fat after drying, (g)

m_2 is the weight of empty flask, (g)

m_0 is the weight of sample, (g)

A.4 Moisture

A .4.1 Principle

The weight lost during heating in an oven at a temperature of 100 ° C. Weight loss or water content is calculated by gravimetry.

A .4.2 Apparatus

a) dessicator;

b) oven;

c) analytical balance;

d) nickel, platinum or aluminum cup with lid.

A .4.3 Determination

a) place carefully 2 g of sample in a cup with lid of known weight, close and weigh;

b) dry the cup containing the sample in an oven at a temperature of (100 ± 2) ° C in open condition until its weight is constant;

c) cover the cup while still in the oven and transfer to the dessicator, cool for 20 minutes to 30 minutes and then weigh it;

d) calculate the water content in the sample.

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A .4.4 Calculation

$$\text{Water content} = \frac{(m_1 - m_2)}{m_0} \times 100\%$$

Where:

m_0 is the weight of sample, (g);
m_1 is the weight of sample before drying, (g);
m_2 is the weight of sample after drying, (g).

A .5 Fineness

A .5.1 Principle

Degree of fineness of samples is measured using a 200 mesh sieve.

A .5.2 Apparatus

a) glass flask;
b) stirrer bar;
c) sieve (200 mesh);
d) Erlenmeyer suction;
e) Whatman 41 filter paper;
f) analytical balance;
g) 103-105 ° C oven;
h) Wash bottle;

A .5.3 Reagents

a) Aceton p.a
b) Detergent (concentrate-surface active agent)

A .5.4 Determination

a. weigh approximately 10 g sample in a glass flask. Add 1 g detergent and 20 ml of hot distilled water and stir carefully with a stirrer rod until there are no more lumps, then add 280 ml hot distilled water (75 ± 5) ° C and stir vigorously for 2 minutes using a mixing mechanical mixer.

b. pour the suspension to a sieve (200 mesh) and shake. Wash the glass flask and filter with 1.5 L of hot distilled water (75 ± 5) ° C. If the suspension does not pass the sieve, tap the sieve slowly until no particles escape.

c. Rinse the sieve and residue with 15 ml to 25 ml of aceton to remove residual water and fat.

d. transfer the filtrate to the watch glass and dry for 45 minutes at a temperature of 103 to 105 ° C.

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e. cool in a dessicator for 45 minutes

f. weigh filter paper, residue, and watch glass.

A .5.5 Calculation

$$\text{residue levels (\%) of fat-free dry material} = \frac{m_1 - m_2}{m_0} \text{ X } 100\%$$

Fineness (%) = 100% - the level of residues

Description:
M_1 is the weight of filter paper, dried residue and watch glass (g);
M_2 is the filter paper and watch glass (g);
M_0 is the weight of sample, (g);

A .6 Shell content was calculated from the alkali free nib

A .6.1 Principle

After destruction organic compounds by a process that does not result in the destruction of hard cells, the residues are suspended in glycerol.

The number of grouped cells in suspension and the number of cells in each group are counted. Content of the skin is calculated by empirical determination from the calibration curve that gives the relationship between the number of hard cell groups and the average size of the cocoa powder with 1% skin content measured in fat-free dry material.

A .6.2 Apparatus

a) 200 ml Erlenmeyer;
b) 50 ml centrufuge tube;
c) Centrifuge;
d) Glass tube, approximately 5 mm in diameter with 1 mm long pointed-tip, length 150 mm;
e) Glass mat measuring approximately 26 x 76 x 1.0 mm;
f) "Counting grid" measuring approximately 20 x 26 x 0.5 mm with a size of 1 mm^2 area.

A .6.3 Reagents

Belluci reagent:
Glacial acetic acid + concentrated HNO_3 + H_2O = 36 + 5 + 9

A .6.4 Determination

a) Weigh approximately 0.5 g fat-free dry powder sample (dried for 1 hour at a temperature of 100 ° C) and place in a 150 ml glass beaker. Add 20 ml of Belluci reagent, stirr gently boil for 10 minutes above a small flame with periodic stirring and cool.
b) Weigh culture tube in 30 ml glass beaker for support. Gradually move the residue into the culture tube with a small amount of water, centrifuge for more than 3 minutes with maximum speed. Discard supernatan liquid, add water until it reaches ¾ tube and shake until the residual separate.
c) Discard the liquid again, add glycerol (3 parts glycerol + 2 parts water) until the weight of the tube and the support weight reaches (20 ± 0.03) g exceeding its initial weight. Shake vigorously until completely mixed, immediately transfer to a glass containers containing a

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small magnetic rod. Hold the glass container and let all bubbles disappear (approximately 5-10 minutes).

d) Weigh together the watch glass with scales and glass cover. Stir the liquid using a magnetic stirrer for 1 minute at maximum speed at which no bubbles are formed. Move (0.04 ± 0.01) g fluid to the center of the glass with scale, weigh 0.1 mg. Place a rubber support in the glass tube to prevent evaporation.

e) Place the glass with scales on the microscope with or without top condenser and with a light filter and light scatterer.

f) Count 2 slides by scanning a slide at 100 x and count stone cells larger than ≥ 100 cells at 100 x. Count entire stone cells, either in the form of units and groups, and also all fragmented stone cells ≥ 0.5 cell. Do not count the smaller fragments.

A .6.5 Calculation

Calculation of stone cells

$$S = \frac{84\,C}{17200\,M - C}$$

Where:

C is the number of stone cells in the fluid;

M is alkaline free nib in mg (= 1 000 WD / L);

W is the weight of sample in grams;

D is calculated fluid in grams;

L is the dilution test solution.

A .7 Metal contamination

A .7.1 Determination of lead (Pb) and cadmium (Cd) concentration

A .7.1.1 Principle

Destruct the samples by dry charring at 450°C, followed by dissolving in acid solution. The dissolved metal is calculated using Atomic Absorption Spectrophotometer (AAS) with a maximum wavelength of 228.8 nm for Cd and 283 nm for Pb.

A .7.1.2 Apparatus

a) Calibrated analytical balance with a precision of 0.1 mg;

b) porcelain / platinum / quartz dish with a capacity of 50 ml to 100 ml;

c) electric bath;

d) *Whatman* paper No. 41;

e) calibrated furnace with an accuracy of 1 ° C;

f) Calibrated Atomic Absorption Apectrophotometers and its accessories (Cd cathode lamps and Pb) (preferably using graphite furnace);

g) calibrated pipette with 0.05 ml measuring scale or micro burette;

h) calibrated 50 ml, 100 ml, and 1000 ml measuring flask;

i) 10 ml capacity measuring cup;

j) 250 ml glass beaker; and

k) water bath.

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A .7.1.3 Reagent

a) Concentrated nitric acid solution, HNO_3 (65%, s.g 1.4);
b) Concentrated hydrochloric acid, HCl (37%, s.g 1.19);
c) Nitric acid solution, HNO_3 , 0.1 N;
d) dilute 7 ml 65% HNO_3 with distilled water in a flask measuring 1 000 ml and dilute to the mark line.
e) solution of hydrochloric acid, HCl, 6N;
f) dilute 500 ml 37% HCl with distilled water in a flask measuring 1 000 ml and dilute to the mark line.
g) standard solution of 1 000 µg / ml Cd;
h) dissolve 1.000 g of Cd with 7 ml of concentrated HNO_3 in a 250 ml glass beaker and pour into a 1000 ml measuring flask and dilute with distilled water until the mark line. Alternatively, a standard solution of Cd 1000 µg / ml ready for use, can be used.
i) 200 µg / ml Cd standard solution;
j) pipette 10 ml standard solution of 1 000 µg / ml Cd into a 50 ml measuring flask and then dilute with distilled water to the mark line and then shake. This second standard solution has a concentration of 200 µg / ml Cd.
k) standard solution of 20 µg / ml Cd;
l) pipette 10 ml standard solution of 200 µg / ml Cd into a 100 ml measuring flask and then dilute with distilled water to the mark line and then shake. This third standard solution has a concentration of 20 µg / ml Cd.
m) working standard solution of Cd;
n) pipette into a 100 ml measuring flask each 0 ml, 0.5 ml, 1 ml, 2 ml, 4 ml, 7 ml and 9 ml of 20 µg / ml standard solution and add 5 ml 1 N HNO_3 solution or 6 N HCl, and dilute with distilled water to the mark line and then shake. This working standard solution has a concentration of 0 µg / ml; 0.1 µg / ml; 0.2 µg / ml; 0.4 µg / ml; 0.8 µg / ml; 1.4 µg / ml and 1.8 µg / ml Cd.
o) standard solution of 1000 µg / ml Pb;
p) dissolve 1.000 g of Pb with 7 ml of concentrated HNO_3 in a 250 ml glass beaker and pour into a 1000 ml measuring flask than dilute with distilled water until the mark lines. Alternatively, can use 1 000 µg / ml Pb standard solution ready for use.
q) standard solution of 50 µg / ml Pb; and
r) pipette 5.0 ml of standard solution of 1 000 µg / ml Pb in a 100 ml measuring beaker and dilute with distilled water to the mark line and then shake. This second standard solution has a Pb concentration of 50 µg / ml.
s) working standard solution of Pb;
t) pipette into a 100 ml measuring beaker each 0 ml, 0.2 ml, 0.5 ml, 1 ml, 2 ml ,3 ml and 4 ml of 50 µg / ml standard solution and add 5 ml 1 N, HNO_3 solution or 6 N HCl, and dilute with distilled water to the mark line and then shake. This working standard solution has a concentration of 0 µg / ml; 0.1 µg / ml; 0.25 µg / ml; 0.5 µg / ml; 1.0 µg / ml; 1.5 µg / ml and 2.0 µg / ml Pb.

A .7.1.4 Determination

a) Weigh exactly 10 g to 20 g sample in a porcelain / platinum / quartz cup (m);
b) place the cup containing the sample test on electrical heater and heat gradually until the test sample does not release smoke anymore;
c) continue charring in the furnace (450 ± 5) ° C until the ash turns white, free from carbon;

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d) if the ash is not free yet of carbon showing a grayish color, wet with a few drops of water and add concentrated HNO_3 drop by drop to approximately 0.5 ml to 3 ml;
e) dry the cup on the electric bath and return to the furnace at a temperature of 450 ° C and continue heating until the ash turns white. Addition of concentrated HNO_3 can be repeated if the ashes are still grayish;
f) dissolved the white ash in 5 ml 6N, HCl or 5 ml 1N HNO_3 while heating on an electric heater or water bath for 2 minutes to 3 minutes and pour into a 50 ml measuring flask and top up with distilled water until the mark line (V) , if necessary, strain the solution using *Whatman* No.41 filter paper;
g) prepare a blank solution by addition of reagents and the same treatment as a sample;
h) read the absorption of standard solution and working solution against blank sample using the AAS at a maximum wavelength of about 228.8 nm for Cd and 283 nm for Pb;
i) construct a calibration curve with the metal concentrations (µg/ ml) as the X axis and absorption as Y axis;
j) plot results of sample solutions using the calibration curve and
k) calculated the metals content in the sample.

A. 7.1.5 Calculation

$$\text{Content of metal (mg / kg)} = \frac{C}{m} \times V$$

Description:
C is the concentration of metal from the calibration curve, (µg / ml);
V is the volume of final solution, (ml);
m is the weight of sample (g).

A.7.1.6 Accuracy

The range of standard deviation (RSD) of two test results is maximum 16%. If the RSD is larger than 16%, then the analysis shall be repeated.

A. 7.2 Determination of tin (Sn)

A.7.2.1 Principle

Destruct the sample with HNO_3 and HCl and then add KCl to reduce interference. Read Sn using a AAS with the maximum wavelength of 235.5 nm using flame oxidation of N_2O-C_2H_2

A.7.2.2 Apparatus

a) calibrated analytical balance with an accuracy of 0.1 mg;
b) 250 ml erlenmeyer;
c) electric bath;
d) *Whatman* paper no. 41;
e) calibrated furnace with an accuracy of 1 ° C
f) calibrated AAS and accessories (Sn cathode lamps);
g) calibrated 5 ml and 10 ml capacity measuring pipette with 0.1 scale;
h) calibrated 50 ml, 100 ml, and 1 000 ml measuring flask;
i) 50 ml capacity measuring glass;
j) 250 ml glass beaker;
k) water bath.

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A.7.2.3 Reagent

a) 10 mg / ml K potassium solution;
b) dissolve 1, 91 g KCl in water to obtain 100 ml;
c) concentrated nitric acid, HNO_3
d) concentrated hydrochloric acid, HCl;
e) 1000 mg / l Sn standard solution; and
f) dissolve 1.000 g of Sn with 200 ml of concentrated hydrochloric acid in a 1000 ml measuring flask, add 200 ml of distilled water, cool at room temperature and dilute with distilled water until the mark lines.
g) standard working solution.
h) pipette 10 ml of concentrated HCl and 1.0 ml of KCl solution each into 100 ml measuring flask. Respectively add 0; 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2, 5 ml of 1000 mg / l Sn standard solution and dilute with distilled water until the mark lines. This working standard solution has a concentration of 0 µg / ml; 10 µg / ml; 15 µg / ml; 20 µg / ml and 25 µg / ml Sn.

A.7.2.4 Determination

a) weigh 10 g to 20 g of sample (m) into a 250 ml erlenmeyer, add 30 ml concentrated HNO_3, and allow 15 minutes;
b) heat gently for 15 minutes in the acid cabinet, avoid the occurrence of excessive sparks;
c) continue heating until the remaining volume reach 3 ml to 6 ml or until the sample began to dry at the bottom, avoid the formation of charcoal;
d) lift erlenmeyer from electric bath, add 25 ml of concentrated HCl, and heat for 15 minutes until the formation of Cl_2 fume stops;
e) increase heating and boil until the remaining volume is 10 ml to 15 ml;
f) add 40 ml of distilled water, stir, and pour into a 100 ml measuring flask, rinse the erlenmeyer with 10 ml of distilled water;
g) add 1,0 ml KCl, cool at room temperature, calibrate with distilled water, and filter;
h) prepare blank solution with the addition of reagents and the same treatment as a sample;
i) read absorbance of standard work solution and sample solution against blank sample using AAS with maximum wavelength of 235.5 nm and flame oxidation of N_2O - C_2H_2;
j) construct a calibration curve with the concentration of Sn (µg / ml) as the X axis and absorbance as Y axis;
k) do the test in duplo; and
l) compute Sn content in the sample.

A.7.2.5 Calculation

$$\text{Content of Sn (mg / kg)} = \frac{C}{m} \text{ X } V$$

Where:

C is the concentration of Sn obtained from the calibration curve, (µg / ml);
V is the volume of final solution, (ml);

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M is the weight of sample, (g).

A.7.2.6 Accuracy

The range of standard deviation (RSD) of two test results is maximum 16%. If the RSD is larger than 16%, then the analysis shall be repeated.

A. 8. The arsenic contamination (As)

A. 8 .1 Principle

Sample is destructed using a mixture of sulfuric acid with hydrogen peroxide. Arsenic is immediately determined by the addition of sodium borohydride using an atomic absorption spectrophotometer using hydride system with a 193.7 nm wavelength.

A. 8 .2 Reagents

a) sulfuric acid (1.84);

b) 30% hydrogen peroxide;

c) 5% sodium borohydride ($NaBH_4$) solution in 1% NaOH solution and filtered using 0.45 um porous filter paper;

d) dilute hydrochloric acid (150 ml HCl in 1000 ml of water);

e) arsenic (As^{5+}) standard solution each ml contains 100 µg As

f) dissolve 0.132 g dry As_2O_3 in 20 ml 30% NaOH solution, add slowly while stirring 100 ml of distilled water, then 10 ml of concentrated H_2SO_4, dilute with distilled water to the mark line on 1 000 ml glass beaker (every ml solution contains 0.1 mg As or 100 µg As);

g) 16% potassium iodide solution

A. 8 .3 Apparatus

a) atomic absorption spectrophotometer with hydride kit;

b) 200 g capacity analytic balance with a precision of 0.1 mg;

c) 10 ml, 100 ml, 1 000 ml measuring glass;

d) 250 ml kjehdal flask.

A. 8 .4 Determination

a) carefully weigh up to 2 g 5 g sample into a kjehdal flask containing some boiling stones, detract with 5 ml hydrogen peroxide and 2.5 ml sulfuric acid carefully to obtain a clear solution, if it is not clear yet, add peroxide and reheat. Repeat until the solution becomes clear;

b) cool, add 2 times 5 ml distilled water by heating to form gas / white smoke, cool again, and then pour into a 10 ml measuring flask to the mark line;

c) treat the blank the same as the sample;

d) pipette 2.5 ml solution sample into a special tube for the examination of arsenic using atomic absorption spectrophotometer and add 10 ml 15% HCl, 0.25 ml 16% KI solution, measure the absorbance immediately after addition of 2 ml $NaBH_4$ with a wavelength of 193,7 nm. Do the same to the blank.

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A. 8 .5 Expression of results

$$\text{Arsenic concentration (µg / g)} = \frac{Ac}{Ab} \times C \times \frac{V}{W}$$

Where:

Ac is the absorption of solution samples obtained;

Ab is the absorption of standard solution obtained;

V is the volume of sample solution;

W is the weight of sample, expressed in g;

C is the standard concentration approaching the example.

A.9 The microbial contamination

A. 9 .1 Total Plate Count

A. 9. 1.1 Principle

Mesophyll aerobic bacterial growth after the sample is incubated in a suitable culture for 24 hours to 48 hours at a temperature of (35 ± 1) ° C.

A.9 .1.2 Cultivation and dilution

a) buffer pepton water (BPW);
b) dissolve 25.5 g BPW in 1000 ml of distilled water, sterilize with an autoclave at a temperature of 121 ° C for 15 minutes.
c) plate count agar (PCA);
d) dissolve 22.5 g PCA in 1000 ml of distilled water, sterilize with an autoclave at a temperature of 121 ° C for 15 minutes.

A. 9 .1.3 Apparatus

a) sterilized petri glass (15 mm x 100 mm) or plastic dish (15 mm x 90 mm);
b) 1 ml, 5 ml and 10 ml pipette with scales;
c) resistant silicate glass dilution bottle, with a rubber stopper or threaded plastic cap;
d) water bath with a thermostat to regulate the temperature at (45 ± 1) ° C;
e) incubator cabinet or an incubator (35 ± 1) ° C;
f) autoclaves;
g) bacterial colony counter, dark field model or same type with a light source and gridplate;
h) calibrated oven.

A.9.1.5 Procedure

a) Preparation of samples
b) weigh 50 g test sample in a sterile plastic bag, put aseptically 450 ml of buffer pepton water solution so that 1:10 dilution is obtained, then shake the mixture until it is homogenuous;
c) by using a different sterile pipette, make a dilution level of 10^{-2}, 10^{-3} ,10^{-4} of the prepared samples as needed. Ten times dilution is made by mixing 10 ml of the previous

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level of dilution with 90 ml of diluent substances, all dilution levels are shaken 25 times with an arc length of 30 cm for 7 seconds.

d) shake each dilution bottle to dissolve the sediment in it, then pipette 1 ml of each dilution to be added to a sterile labeled petri dish and do this in duplo;
e) pour 12 ml to 15 ml of plate count agar media of (45 ± 1) ° into each petri dish within 15 minutes after the first dilution was made, for each range of dilution, also pour agar with diluent as inoculum in a petri dish to be used as control;
f) cultivation and the agar meda is mixed thoroughly by moving the petri dish backwards, forwards and rotation on a flat surface;
g) leave to freeze the mixture in the dish;
h) place the petri dish upside down in an incubator cabinet at a temperature of (35 ± 1) ° C for (48 ± 2) hours;
i) count the number of bacteria in each petri dish containing 25 colonies to 250 colonies and record the results for each level of dilution.

A.9.1.6 Calculation

Average the results and report as total number per g of test sample. Total bacterial count per g = average number of colonies per dish multiplied by the dilution factor.

Total plates count = n x f

Where:
n is the average number of colonies from two petri dishes from a single dilution (colony / g);
f is the dilution factor.

A. 9.1.6 .1 Calculation of colonies

a. count the colonies on each petri dish containing 25 colonies to 250 colonies for a single dilution, calculate the average number of colonies and multiply by the dilution factor. Express the result as the number of bacteria per gram. If the results of two dilution is between 25 colonies - 250 colonies, calculate the number of colonies for each dilution by the following equation:

$$N = \frac{\sum C}{[(1 \times n_1) + (0{,}1 \times n_2)] \times (d)}$$

Where:
N is the number of bacteria per gram, (colonies / g);
Σ C is the sum of all colonies counted on a petri dish, (colonies);
n_1 is the number of dishes on the first dilution calculated;
n_2 is the number of petri dishes in the second dilution calculated;
d is the first dilution factor used in the calculation.

Example:

10^{-2}	10^{-3}
232	33
244	28

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$$N = \frac{(232+244+33+28)}{[(1 \times 2) + (0.1 \times 2)] \times (10^{-2})} = 24{,}000$$

b. if there is one or more petri dishes containing more or less than 250 colonies, calculate the number of colonies that lie between 25 colonies to 250 colonies and use the equation in point a);
c. if the number of colonies of each colony that grow on the petri dish is less than 25 then expres the estimate of number of bacteria as less than 25 colonies multiplied by the lowest dilution;
d. if all petri dishes contain more than 250 colonies for all dilutions, note the number of bacteria as too numerous to count (TNTC);

If the number of bacteria is less than 100 colonies per cm^2 with the spread of representative colonies approaching 250, make an estimate of the number of colonies and express the results as an estimated number of bacteria namely number of bacteria multiplied by the dilution factor;

Example:

10^{-2}	10^{-3}	estimated number of bacteria
TNTC	640	640,000 (6.4×10^6)

- if the number of bacteria is more than 100 colonies per cm^2 report the results as > 100 x dilution factor x area;

Examples of average number of 110 colonies per cm^2:

10^{-2}	10^{-3}	area (cm^2)	estimated number of bacteria
TNTC	7150	65	> 6.500.000 ($6{,}5 \times 10^6$)
TNTC	6490	59	> 5.900.000 ($5{,}9 \times 10^6$)

e. if there are no colonies at all at this dilution rate, record, and report as <10. This writing reveals an estimate.
f. All the plates with spreaders and / or because of laboratory accidents. Reported as Spreader (SPR) or Laboratory Accident (LA).

A.9.1.6.2 Calculation and rounding of numbers

In reporting the number of colonies only 2 significant figures are used, namely the first and second digit (starting from left),

a) if the third figure from the left is 5 or greater than 5, then the number is changed to zero and the second number rounded up,
example: 528 is reported as 530, in writing 5.3×10^2
b) if the third number is 4 or less than 4, then the third number is replaced by zero and the second number retained,
for example: 528 to 530, in writing 5.2×10^2
c) if the third number equals 5 then rounded as follows:
 - Rounded up if the second number is an odd number,
 example: 575 is reported as 580, in writing 5.8×10^2
 - Rounded down if the second number is an even number
 example: 565 is reported as 560, in writing 5.6×10^2

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A. 9.2 Determination of *Coliform* bacteria and *Escherichia Coli*

A. 9.2 .1 Principle

Coliform bacteria growth characterized by the formation of gas in Durham tubes, after the test samples are incubated in an appropriate cultivation media at a temperature of (35 ± 1) ° C for 24 hours until 48 hours later referred to Table MPN (Most Probable Number).

A.9.2.2 Cultivation and dilution

a) buffer pepton water (BPW);

 Dissolve 25.5 g of BPW in 1000 ml of distilled water, sterilize in an autoclave at a temperature of 121 ° C for 15 minutes.

b) Lauryl Sulphate Trytose (LST) Broth;

c) 2% Brilliant Green Lactose Bile (BGLB) Broth;

d) E.C Broth;

e) Levine's Eosin methylene blue agar (L-EMB);

f) Plate count agar (PCA);

A.9.2.3 Apparatus

a) autoclave

b) 1ml, 5 ml 10 ml pipette with scales;

c) resistant silicate glass dilution bottles, with a rubber stopper or plastic threaded cap;

d) water bath with a thermostat to regulate the temperature at (45 ± 1) ° C;

e) incubator cabinet or incubator of 35 ± 1 ° C;

f) autoclaves;

g) inoculation needle (ose) with a diameter of approximately 3 mm;

h) test tube and Durham tube;

i) test tube rack.

A.9.2.4 Determination

A.9.2.4.1 MPN - Presumtive test for coliform bacteria and E. coli (allegation test)

a) Sample preparation in accordance with A.9.1.5 a), b);

b) Inoculate 1 ml solution of each level of dilution to three tubes containing lauryl sulfate broth. Hold the pipette so that the lower end of a pipette touches the tube, let the content of the pipette flow for 2 seconds to 3 seconds. Do not blown pipette to release its content;

c) insert these tubes into the incubator at a temperature of 35 ° C.

d) observe the tubes after (24 ± 2) hours, if there is a tube that already contains gas then the tube is positive;

e) the tubes not containing any gas yet are declared as negative tubes, continue the incubation for 24 hours;

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f) note the existence of gas formation after incubation of (48 ± 2) hours and declared the tubes as 'positive';

g) do a confirmation test for all positive tubes in the presumtive test;

A.9.2.4.2 MPN - Confirmed test for coliform bacteria (confirmation test)

a) move one eye ose from each positive LST tube into the a different 2% BGLB tube;
b) incubate 2% BGLB tubes for 48 hours ± 2 hours at a temperature of (35 ± 1) ° C;
c) record all the positive tubes BGLB produces gas and by using tables Numbers Most Likely (APM) Table A.1, specify the APM based on the number of tube formation BGLB showing any amount of gas, during (48 ± 2) hours at (35 ± 1) ° C;
d) report as MPN coliform bacteria per g

A. 4 .2.4.3 MPN - Confirmed test for Escherichia coli (confirmation test)

a) move one ose from each positive LST tube into the a different tube of EC Broth;
b) incubate the EC tubes for ± 24 hours at 45.5 ° C, tubes containing gas is are positive tubes;
c) if negative, incubate and check again the (48 ± 2)^{ed} hour, if gas has formed the tube is positive;

A.9.2.4.4 MPN - Complete test for Escherichia coli (complete test)

a) Shake the EC positive tubes carefully;
b) scratched / plant in a L-EMB agar dish to produce separate colony with minimum distance of 0.5 cm;
c) incubate these L-EMB dishes for (18 - 24) hours at (35 ± 1) ° C;
d) check the dishes for brown / greenish colonies with or without a metal glosh;
e) from every L-EMB dish, move a maximum of 5 suspicious colonies on the PCA slanted tube;
f) incubate the slanted agar tubes for (18 - 24) hours at (35 ± 1) ° C and use for consecutive tests;
g) make coloring g of each E. coli culture, is gram negative and rod-shaped without spores tested using the IMViC reactions as mentioned below and should be inoculated again in LST tubes to confirm the existence of gas formation;

- Formation of Indol,

Inoculate tryptone broth tube, incubation for (24 ± 2) hours at (35 ± 1) ° C, test the presence of indol by adding (0.2 to 0.3) ml Kovacs reagents. This test is positive when the top layer is red.

- Voges Proskauer and Methyl red reaction

Inoculate MR-VP tube from each PCA tube and incubate for (48 ± 2) hours at 35 °C, aseptically transfer of 1 ml culture to a sterile test tube. Add 0.6 ml of 5% alpha naftol solution in alcohol, 0.2 ml 40% KOH solution and several creatine crystals, stir and leave for 2 hours. The test is positive if the Eosin color change to pink. MR -VP tube that was initially incubated for (48 ± 2) hours at (35 ± 1) ° C, add 5 drops of methyl red indicator to each tube, the culture is positive MR if there appears a red color, negative MR when the color is yellow.

- Use of citrate

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Carefully inoculate Koser's citrate tube, incubate for 96 hours at (35 ± 1) °C, the reaction is positive when clouding occures. jika terbentuk kekeruhan. Carefully tube Koser's citrate medium inoculated, incubated for 96 hours at a temperature (35 ± 1) ° C, the reaction is positive when the turbidity is formed.

- The formation of gas from lactose

Inoculate LST tubes for each slanted PCA agar, incubate for (48 ± 2) hours at a temperature of (35 ± 1) ° C, check the tubes for gas formation.

h) Classify as E. coli when IMViC with indol formation, Methyl red, Voges Proskaeur and Citrat are ++ (biotype 1) or ---.(biotype 2).

- In addition to IMViC test, API20E or Vitek automated biochemical assay can be used for the identification of E. coli.

i) Count the MPN E. coli using a MPN table based on the number of tubes of 3 series of dilution that certainly contain E. coli.

Table A. 1 - MPN per 1 g sample when using 3 tubes for each level of dilution (0.1 g / ml; 0.01 g / ml and 0.001 g / ml) samples

Positive tubes			MPN/ g	Tolerance Limit		Positive tubes			MPN / g	Tolerance Limit	
0,1	0,01	0,001		Low	High	0,1	0,01	0,001		Low	High
0	0	0	<3.0	- --	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8,7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1.000
2	0	2	20	4.5	42	3	3	0	240	42	1.000
2	1	0	15	3.7	42	3	3	1	460	90	2.000
2	1	1	20	4.5	42	3	3	2	1100	180	4.100
2	1	2	27	8.7	94	3	3	3	> 1100	420	- --

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A.9.3 Salmonella

A.9.3.1 Principle

Sample tested are cultivated in an enriched media and grown on selective media. Next detect by growing on a selective agar media, then isolated of colonies suspected as Salmonella and followed by confirmation by biochemical tests and serological tests to ensure there existence or absence of Salmonella bacteria.

A.9.3.2 Cultivation and reagents

a) Lactose Broth;
b) Nonfat dry milk (reconstituted)
c) Tetrathionate Broth (TTB);
d) Brain Heart Infusion (BHI) Broth
e) 1% Brilliant Green Dye Solution
f) Rappaport Vassiliadis (RV) medium;
g) Bismuth Sulfite Agar (BSA);
h) Hectoen Enteric (HE) Agar;
i) Triple Sugar Iron (TSI) Agar;
j) Xylose lysine desoxycholate (XLD) Agar;
k) Lysine Iron Agar (LIA);
l) MacConkey Agar;
m) Urea Broth;
n) Lysine Decarboxylase Broth (LDB)
o) Tryptone (tryptophane) broth;
p) trypticase soy-tryptose broth;
q) 0.2% Bromcresol purple dye solution;
r) Urea Broth;
s) rapid urea broth;
t) malonate broth;
u) 40% potassium hydroxide solution;
v) Kovacs reagent;
w) Simmons citrat agar;
x) MR-VP Broth;
y) Phenol red lactose;
z) Purple lactose broth;

A.9.3.4 Apparatus

a) calibrated scale, with a sensitivity of 0.1 mg;
b) incubator (35 ± 1) ° C;
c) sterile spoons;
d) petri, glass or plastic dish, 15 mm x 100 mm or 15 mm x 90 mm;
e) 1 ml and 10 ml pipette with 0.01 ml, 0.1 ml, scales;
f) inoculation needle with internal diameter of approximately 3 mm (ose);
g) sterile plastic bags 28 cm x 37 cm, which can be closed / sealed again.;
h) test tubes and rack;

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i) oven;
j) water bath;
k) glass stirrer;
l) diluent vial
m) pH meter
n) Bunsen

A.9.3.5 Determination

A.9.3.5.1 Sample preparation and pre-enrichment

a) weigh 25 g sample into a sterile blender tube;
b) add 225 ml of sterile reconstituted non-fat milk, shake for 2 minutes;
c) aseptically transfer to a sterile 500 ml bottle, leave at room temperature for (60 ± 5) minutes with the bottle closed;
d) add 0.45 ml of 1% Brilliant Green dye solution and shake until homogenuous;
e) loosen the bottle lid, or by ¼ thread rotation;
f) incubate at 35 ° C for (24 ± 2) hours;

A.9.3.5.2 Enrichment

a) Tighten the lid and gently shake the incubated samples;
b) add 0.1 ml pre-enrichment culture into 10 ml Rappaport-Vassiliadis (RV) medium and 1 ml to 10 ml tetrathinate (TT) broth;
c) incubate RV at (42 ± 0.2) ° C for (24 ± 2) hours and TT broth (35 ± 0.2) ° C for (24 ± 2) hours

A.9.3.5.3 Planting and selection / selective cultivation

a) by using circular 3 mm ose needles (10µl), put scratched cultures from the TT enriched media into a petri dish containing XLD, HE and BS agar media. Prepare BS agar one day before use and store in a dark place at room temperature until ready to be scraped;
b) repeat the above steps for the RV enrichred media;
c) incubate for (24 ± 2) hours;
d) observe for possible presence of Salmonella colonies.

- Salmonella colony morphology:

Take 2 or more Salmonella colonies from each selective agar media after (24 ± 2) hours of incubation. Typical Salmonella colonies are as follows:

HE: colonies of blue-green to blue color with or without a black core. Most Salmonella form large colonies, shiny black core, or perhaps nearly all black. A small section of Salmonella culture form yellow colonies with or without a black core in HE and XLD media.

XLD: pink colonies with or without a black core. Most Salmonella form large colonies, shiny black core, or perhaps nearly all black.

BS: colonies of brown, gray to black color, sometimes shiny like metals. If the incubation period is extended then the color of the surrounding media changes from brown to black (halo effect). In some strains the colonies are green with or without a dark color in the surrounding media.

- If there are no typical colonies or suspected colonies in BSA media after incubation for (24 ± 2) hours, continue incubation for (24 ± 2) hours. If there are no suspect colonies after (48 ± 2) hours of incubation, take 2 or more colonies that are not typical;

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e) carefully take the center of a colony with a sterile ose needle, inoculate in TSI agar media by scratching the slanted agar and piercing the straight agar. Without taking new colonies inoculate with the same ose needle in the LIA media by piercing the straight agar twice after that scratch the slanted agar. Since the Lysine decarboxylase reaction is anaerobic the slanted LIA shall have have a deep puncture (4 cm).; Save the selected selective agar media at a temperature of (5 - 8) ° C;

f) Incubate TSI and LIA at temperature of 35 ° C for (24 ± 2) hours with the lid slightly loose conditions to prevent the formation of excessive H_2S.

TSI : typical Salmonella cultures give an alkaline reaction (red) on the scratches and acid reaction (yellow) at the puncture, with or without H_2S gas evolution (blackish color of agar).

LIA : typical Salmonella cultures give an alkaline reaction (purple) in the whole tube. Truely yellow reaction in the puncture is stated as a negative culture. In general, Salmonella cultures produce H_2S in LIA and some non-Salmonella cultures form a brick red reaction in the slanted LIA agar.

g) All cultures considered as potential Salmonella and subjected to biochemical and serological tests are as follows:

- Give an alkaline reaction at the puncture in the LIA media regardless of the TSI reaction;
- Give an acid reaction at the puncture in the LIA media and an alkaline reaction at scrathes and acid reaction at TSI puncture.

Cultures that provide acid to the LIA media and acid at scrathes and acid reaction at punctures in TSI can be stated as non Salmonella.

If the TSI cultures do not show typical Salmonella reactions (alkaline at scrathes and acid at punctures), repeat the test by taking a suspicious colonies from a selective media that does not provide suspected positive culture and inoculate by scratching TSI and LIA media as in the mehod shown in sub clause e)

h) Perform biochemical and serological identification tests of:

- three presumtive TSI culture of 1 set of selective media (HE, XLD, and BSA) that are inoculated from TTB, and three presumtive cultures inoculated from RV;
- if three presumtif positive TSI cultures from different agar media, test at least 6 TSI cultures for each 25 g of food samples.

A.9.3.6 Identification of Salmonella

A.9.3.6.1 Mixed culture

a) When TSI agar culture appears to be mixed, then scratch again in MacConkey, HE or XLD agar media. Incubate for (24 ± 2) hours at a temperature of 35 ° C. Observe the colonies suspect to be Salmonella:

a. MacConkey agar.

Typical colonies appear transparent and colorless, sometimes with a black core. Salmonella colonies will form bright areas due to precipitation by other bacteria that sometimes grows;

b. Hektoen enteric (HE) agar

Blue-green to blue colonies with or without a black core. Salmonella culture in general will form large colonies, shiny black core or almost all the colonies appear black;

c. Xylose Lysine dexoxycholate (XLD) agar

Pink colonies (pink) with or without a black core. Salmonella culture in general form large colonies, shiny black core or almost all colonies are black.

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b) Transfer at least 2 suspected Salmonella colonies on TSI and LIA media in accordance with Article A.9.3.5.3 e and f.

A.9.3.6.2 Pure culture

a) urease test (conventional)

Inoculate from TSI suspected Salmonella using needle inoculation into the Urea Broth. Incubate for (24 ± 2) hours at a temperature of 35 ° C;

b) urease test (fast)

Inoculate from TSI suspected Salmonella using needle inoculation into the rapid urea broth. Incubate for 2 hours in a water bath at a temperature of (37 ± 0.5) ° C. A typical Salmonella reaction for the urease test gave a negative result (no color change);

A.9.3.6.3 Polyvalent flagellar serological test (H)

a) Inoculate from each TSI agar giving a negative urease reaction into:
 - BHI broth, and incubate for 4 hours to 6 hours at a temperature of 35 ° C until visible growth is observed (to be tested on the same day) or
 - Trypticase Soy Trypticase broth (TSTB) and incubate for (24 ± 2) hours at a temperature of 35 ° C. (to be tested the next day). Add 2.5 ml of physiological formanilized saline solution to 5 ml of culture mentioned above;

b) Prepare 2 cultures of TSI 2 (sample and control) that has been given physiological formanilized saline Salmonella polyvalent flagellar (H) antisera. Add ± 0.5 ml formanilized antigen. Incubate the mixture in a water bath at a temperature of 48 ° C to 50 ° C. Examine at every 15-minute interval and observe the results during 1 hour.
 - Positive if clotting occures in the test mixture and no clotting in the control.
 - Negative if no clotting occures in the test mixture and control.
 - Non-specific, if clotting occures in the test mixture and control.

A.9.3.6.4 Urease negative culture test

a) Lysine Decarboxylase broth (LDB)

This test is only applied if the LIA reaction is in doubt.

- Take 1 ose from the Salmonella suspected slanted TSI agar scratch. and inoculate in LDB media. Loosen the lid and incubate for (48 ± 2) hours at a temperature of 35 ° C, but observed at intervals of 24 hours. Salmonella give an alkaline reaction indicated by the color purple in the whole media. Negative reactions are shown by a yellow color in the whole media. If the reaction results show neither a yellow or purple color add a few drops of 0.2% bromocresol purple dye and observe the color changes;
- Phenol red dulcitol or purple broth base with 0,5 % dulcitol
- Inoculate dulcitol media broth from TSI culture. Loosen the lid and incubate for (48 ± 2) hours at a temperature of 35 ° C, but observed after 24 hours. Generally Salmonella give positive results indicated by the formation of gas in Durham tubes and acid pH (yellow) in the media. Negative reaction are characterized by the absence of gas evolution in Durham tubes and phenol red as indicator or bromocresol purple as indicator in the whole media.

c) Tryptone (trytophane) broth (TB)

Inoculate tryptone broth media with TSI cultures. Incubate for 24 hours at a temperature of 35 ° C and then follow the following procedure:

- Potassium Cyanida (KCN) Broth

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- Transfer 1 ose cultures of 24 hours TB to KCN broth media. Close the tube tightly and cover with parafilm paper. Incubate for (48 ± 2) hours at a temperature of 35 ° C but observed after 24 hours. Positive results are shown by growth (indicated by turbidity). Generally Salmonella does not grow in this media indicated by non occurrence of turbidity .
- Malonate broth
- Move 1 ose of TB culture into the Malonate broth media. Incubate for (48 ± 2) hours at a temperature of 35 ° C but observed after 24 hours. Sometimes the Malonate broth tube not inoculated turn blue (positive test), therefore Malonate broth is used as control. Generally Salmonella give a negative reaction (green or no color change) in this broth.
- Indol test
- From the remaining TB media, add 0.2 ml to 0.3 ml of Kovacs reagent, immediately observe after addition of reagents. Positive reaction is indicated by the formation of a red ring on the surface of the media. Generally Salmonella gives a negative reaction (no red ring formed on the surface of the media). A color reaction between orange and pink is declared as a positive reaction.

d) The culture is non Salmonella if the Indol reaction is positive and Flagellar (H) negative, or KCN positive and LBD negative.

A.9.3.6.5 Somatic (O) serological test

a) by using a wax pencil (crayon), create two squares each measuring 1 cm x 2 cm on a glass or plastic petri dish of size 15 mm x 100 mm glass-scale object;
b) emulsify i loop round ose slanted TSI cultures of 24 hours to 48 hours with 2 ml 0.85% saline (cultures of tryptose blood agar base without blood can also be used);
c) add 1 drop of suspension cultures on each rectangular section which has been marked with a wax pencil;
d) add 1 drop of saline solution on the first section and add 1 drop of polyvalent somatic (O) antiserum to the other parts;
e) combine or homogenize each section using a clean and sterilized ose for 1 minute;
f) Shake gently for 1 minute and observed using a dark background and good lighting.
g) classification by polyvalent somatic (O) test shows the following results:
 - Positive, if it clotting occurs in a test mixture and no clotting, of the saline control.
 - Negative, if no clotting occures in the test mixture and the saline control.
 - Non-Specific, if clotting occures in the test mixture and the saline control.

A.9.3.6.6 Additional biochemical tests

The cultures that provides an unique reaction in accordance with Table A.2 are stated as Salmonella. If 1 TSI culture from each 25 g sample showed Salmonella, additional biochemical tests for TSI cultures different from the 25 g sample is not necessary. Culture that gave positive reactions in flagellar serological (H) tests but did not show characteristics of Salmonella in biochemical tests, shall be purified according to Clause A.5.6.1 and Clause A.5.6.2

Perform the following additional test for cultures that does not give typical reactions as shown in Table A.3.

a) Phenol red lactose or purple lactose broth

Inoculate broth with a slanted TSI agar culture that has been incubated for 24 hours - 48 hours. Incubate for (48 ± 2) hours at a temperature of 35° C, but observed after 24 hours;

- Positive, if there is acid formation (yellow) and gas in Durham tubes.

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If only acid formation occurs, it can be stated as positive. Salmonella generally gives negative results, indicated by no gas formation in the Durham tube and red (phenol red as indicator) or purple (bromcresol purple as indicator) in the whole media.

- Declare as non Salmonella if the culture gave a positive lactose reaction, except for the culture that gave acid reactions on the slanted TSI agar and an alkaline reaction in LIA or positive reaction in malonate broth. Apply further tests on these cultures to determine whether these cultures are S. Arizonae.

b) Phenol red sucrose or sucrose purple broth

Follow the procedure in accordance with point a), declare it as non Salmonella in a culture that gives a positive sucrose reaction, except a culture that gives an acid reaction to slanted TSI agar and positive reaction in LIA;

c) Methyl Red-Voges-Proskauer (MR-VP) broth

Inoculate a media with a small amount of slanted TSI agar culture and incubate for (48 ± 2) hours at a temperature of 35 ° C

Perform *Voges-Prokauer* (VP) test at room temperature as follows:

- Place 1 ml MR-VP broth that has been incubated for (48 ± 2) hours into a sterile reaction tube and incubate the MR-VP broth again for (48 ± 2) hours at a temperature of 35 ° C.
- Add 0.6 ml alpha naftol and shake.
- Add 0.2 ml of 40% KOH solution and stir again. To accelerate the reaction add a small amount of creatin crystals and observe the results after 4 hours..
- Change in color from brick red to ruby red in the media indicates a positive reaction. Generally Salmonella gives a negative VP reaction indicated by the absence of any change in color from pink to red in the whole media..

Methyl Red (MR) test

- Add 5 drops of methyl red indicator to the MR-VP media that has been incubated for 96 hours.
- Immediately observe the results
- Generally Salmonella gives a positive reaction, indicated by the diffusion of red color in the media. A yellow color indicates the occurrence of negative reaction. If the culture yields a positive KCN and VP reaction and a MR VP negative reaction, then it is stated as non Salmonella

d) Simmons citrate agar

- Inoculate the media by using a needle containing cultures of slanted TSI agar, by scratching the slanted agar and piercing straight agar. Incubate for (96 ± 2) hours at a temperature of 35 ° C.
- Positive, if growth occures that is usually accompanied with color changes from green to blue. Salmonella generally gives positive citrate results.
- Negative indication, if no or very little growth occures and no color changes.

A.9.3.6.7 Expression of results

Report as Salmonella those cultures that indicate reactions shown in Table A.2. Report as non Salmonella those cultures that indicate reactions shown in Table A.3. If there is no single culture indicating Salmonella reaction on biochemical tests, apply biochemical tests starting

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from clause A.5.6.3 to cultures giving negative ureas reaction on the same sample.

Table A. 2 - Biochemical and serology reaction of Salmonella

Test or substrate	Results		Salmonella species reaction
	Positive	Negative	
Glucose (TSI)	yellow puncture	red puncture	+
Lysine Decarboxylase (LIA)	purple puncture	yellow puncture	+
H_2S (TSI and LIA)	black	not black	+
Urease	purple to red	no color change	-
Lysine Decarboxy broth	Purple	Yellow	+
Phenol Red Dulcitol broth	Yellow color with / without gas	Without the formation of gas, no color change	b
KCN broth	growth	No growth	-
Malonate Broth	Blue	No color change	- [c]
Indol test	violets color of surface	Yellow surface	-
Polyvalent flagellar test	clotting	No clotting	+
Polyvalent somatic test	clotting	No clotting	+
Phenol red lactose broth	Yellow color with / without gas	No formation of gas and no change in color	- [c]
Phenol red sucrose broth	Yellow color with / without gas	No formation of gas and no change in color	-
Voges-prokquer test	Pink to red	No change in color	-
Methy red test	Red spreading	Yellow spreading	
Simmons citrate	Growth, blue	no growth and no change in color	V

Notes:
[a] 90% or more positive in one or two days;
- 90% or more positive in one or two days;
V variable
b majority of Arizona culture, negative
c majority of Arizona culture, positive

Table A. 3 - biochemical reaction and serology for the *non-Salmonella*

Test or substrate	Results
Urease	Positive (purple-red color)
Indole test and Polyvalent flagellar (H) test	Positive (violet color on the surface), Negative (no clotting)
Lysine decarboxylase and KCN	Negative (yellow), positive (growth)

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Phenol red lactose broth	Positive (yellow color with / or without gas) (a), (b)

Table A.3 (continued)

Test or substrate	Results
Phenol red sucrose broth	Positive (yellow color with / or without gas) (b)
KCN broth, Voges-Proskauer test and Methyl red test	Positive (growth), positive (pink-red), negative (yellow color spread)

Notes:

a is a positive malonate broth test to be examined further if the cultures are Salmonella arizonate

b is not to disposed of positive cultures if the LIA cultures show reactions characterizing Salmonella, continue testing to observe the species of Salmonella.

As an alternative system to the conventional biochemical tube, 5 commercial biochemical systems (API 20E, Enterotube II, Enterobacteriaceae II, MICRO-ID, or Vitek GNI) can be applied to generic identification of Salmonella. These commercial systems are selected based on the required analysis. These commercial biochemical test can not be used as a substitute for the serological test.

A.9.4 Determination of molds and yeasts

A.9.4.1 Principle

Growth of mold and yeast in appropriate media, after incubation at a temperature of (25 ± 1) °C or room temperature for 5 days.

A.9.4.2 Cultivation and dilution

a) buffer pepton water (BPW);

 dissolved 25.5 g BPW in 1 000 ml of distilled water, sterilize in an autoclave at a temperature of 121 ° C for 15 minutes.

b) potato dextrose agar or other seeding media such as malt extract agar or plate count agar with added chloramphenicol antibiotic (100 mg / liter).

 Dissolve 39 g of PDA in 1 000 ml distilled water, sterilize in an autoclave at a temperature of 121 ° C for 15 min, pH of 3.5 is regulated with 10% sterile tartrate acid, or possibly by adding 100 mg / liter chloramphenicol antibiotic.

A.9.4.3 Apparatus

a) sterilized petri glass dish (15 mm x 100 mm) or plastic dish (15 mm x 90 mm);
b) 1 ml, 5 ml and 10 ml pipette with scales;
c) resistant silicate glass dilution bottles, with rubber stoppers or threaded plastic cap;
d) water bath with a thermostat to regulate the agar temperature at (45 ± 1) ° C;
e) calibrated incubator cabinet or an incubator (25 ± 1) ° C,;
f) autoclaves;
g) bacterial colony counter

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h) calibrated oven.

A.9.4.4 Procedure

a) Sample preparation

weigh 50 g test sample into a sterile plastic bag, pour aseptically 450 ml of diluent solution (buffer pepton water) so that a dilution of 1:10 is obtained, then stir until the mixture is homogen;

b) Using a different sterile pipette, prepare a dilution level of 10^{-2} ,10^{-3} ,10^{-4} of the prepared samples as needed. To prepare a ten times dilution is by mixing 10 ml of the previous level of dilution with 90 ml of diluent substances, all dilution levels are shaked 25 times with an arc length of 30 cm for 7 seconds.

c) shake each dilution bottle to dissolve the sediment, then pipette 1 ml of each dilution into a sterile petri dish that has been labeled and make the test in duplo;

d) pour (12 -15) ml of PDA media at a temperature of (45 ± 1) °C into each petri dish within 15 minutes after the first dilution was made, for each range of dilution, also pour agar with diluent substance as inoculum in a petri dish to be used as control;

e) seeding and agar media are mixed thoroughly by moving the petri dish backwords, forwards and rotation on a flat surface;

f) leave the mixture in the dish to solidify;

g) place the petri dish in an incubation cabinet in reversed position at a temperature of (25 ± 1) ° C for 5 days;

h) count the number of mold / yeast colonies on each petri dish (the counting can be started from the third day to the fifth day), colonies of mold usually are blurred and fluffy while yeast colonies are white and slippery (acid smell), affirm the colony by microscope examination to ensure whether the colony is mold or yeast.

Express the count results as the number of mold / yeast per gram of sample

A.9.4.5 Expression of results

a) count the number of colonies on each petri dish containing 10 to 150 colonies using a colony counter;

b) calculate the average number of colonies and multiply by the dilution factor;

c) express the results as the number of mold / yeast per gram

d) if there are no colonies on a petri dish, report mold / yeast count as a <10

A.9.4.6 Calculation and rounding of numbers

a) if the third figure from the left is 6 or greater than 6, then the number is changed to zero and the second number rounded up,
example: 456 is reported as 460, in writing $4{,}6 \times 10^{2}$

b) if the third number is 4 or less than 4, then the third number is replaced by zero and the second number retained,
example: 454 is reported as 450, in writing 4.5×10^{2}

c) if the third number equals 5 then rounding is as follows:
- rounded up if the second number is an odd numbers,
 example: 455 is reported as 460, in writing 4.6×10^{2}
- Rounded down if the second number is an even number
 example: 445 is reported as 440, in writing 4.4×10^{2}

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BADAN STANDARDISASI NASIONAL - BSN
Gedung Manggala Wanabakti Blok IV Lt. 3,4,7,10
Jl. Jend. Gatot Subroto, Senayan Jakarta 10270
Telp: 021- 574 7043; Faks: 021- 5747045; e-mail : bsn@bsn.go.id

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